

AD \_\_\_\_\_

Award Number: DAMD17-00-1-0411

TITLE: Breast Cancer Immunotherapy with Intra-Tumoral Injection  
of Genetically Modified Dendritic Cells

PRINCIPAL INVESTIGATOR: Seon-Hee Kim, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh  
Pittsburgh, Pennsylvania 15260

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

**BEST AVAILABLE COPY**

**20040802 039**

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> July 2003	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Jul 00-30 Jun 03)	
<b>4. TITLE AND SUBTITLE</b> Breast Cancer Immunotherapy with Intra-Tumoral Injection of Genetically Modified Dendritic Cells			<b>5. FUNDING NUMBERS</b> DAMD17-00-1-0411	
<b>6. AUTHOR(S)</b> Seon-Hee Kim, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Pittsburgh Pittsburgh, Pennsylvania 15260  E-Mail: seonhee@pitt.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> We have hypothesized that intra-tumoral injection of immature DCs should result in acquisition of tumor antigens at the site of injection followed by migration to lymph node and spleen. Here we have used adenoviral vectors, able to efficiently infect both murine and human DC, to deliver IL-12, IL-18, and GM-CSF and the costimulatory molecules B7.1 and CD40L, either alone or in combination, to bone marrow-derived DC (BmDC). We showed that the genetic modification of DCs to overexpress IL-12, GM-CSF, and CD40L significantly enhanced their ability to stimulate a systemic immune response following intra-tumoral injection. In addition, we have used adenoviral gene transfer of members of the TNF family able to stimulate apoptosis, FasL and TRAIL, to render DC direct, potent mediators of tumor cell apoptosis. We demonstrate that the genetic modification of DCs by adenoviral infection to overexpress IL-12, IL-18, and GM-CSF significantly enhanced their ability to stimulate a systemic immune response in the murine breast tumor TS/A following intra-tumoral injection. Furthermore, expression of FasL and TRAIL on DC resulted in a stronger anti-tumor response following intra-tumoral delivery. The anti-tumor effect of different genetically modified-DC was mediated, in part, by the induction of specific CTL activity against the tumor. The genetic modification DC to express one or more immuno-stimulatory molecules was able to augment NK cells and CTL cytolytic activity and IFN-gamma production for subsequent immune anti-tumor responses locally as well as systemically. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. Our research is expected to lead Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.				
<b>14. SUBJECT TERMS</b> Gene therapy, gene delivery and expression, immunology, dendritic cell biology, cytokines, co-stimulatory molecules				<b>15. NUMBER OF PAGES</b> 32
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

## Table of Contents

Cover .....	1
SF 298.....	2
Table of contents .....	3
Introduction .....	4
Body .....	5
Tables .....	9
Key Research Accomplishments .....	10
Reportable Outcomes .....	10
Conclusions .....	11
References .....	13
Figures .....	14
Appendices .....	31

## INTRODUCTION

We have hypothesized that intra-tumoral injection of immature dendritic cells (DCs) should result in acquisition of tumor antigens at the site of injection followed by migration to lymph node and spleen. Indeed, we have demonstrated previously that fluorescently labeled DCs are able to migrate to lymph nodes and spleen following intra-tumoral injection and stimulate a tumor specific T cell response (1). Systemic delivery of BmDC promotes prophylactic and therapeutic anti-tumor immunity (2-6). Furthermore, immature DC injected intra-tumorally (IT) acquire tumor-associated antigen (TAA) in situ and then process and present the captured antigens in lymphoid organs. Presentation of these antigen naïve T cells should efficiently initiate a tumor specific immune response (7,8). The IT injected DCs are able to migrate to lymph nodes where they can present TAA acquired at the tumor site. Here we have used adenoviral vectors, able to efficiently infect both murine and human DC, to deliver interleukin-12 (IL-12), interleukin-18 (IL-18), and granulocytes and macrophages colony stimulating factor (GM-CSF) and the costimulatory molecules CD80 (or B7.1) and CD40 ligand (CD40L), either alone or in combination, to bone marrow-derived DC (BmDC). We showed that the genetic modification of DCs to overexpress IL-12, GM-CSF, and CD40L significantly enhanced their ability to stimulate a systemic immune response following intra-tumoral injection. In addition, we have used adenoviral gene transfer of members of the tumor necrosis factor (TNF) family able to stimulate apoptosis, FasL and TNF-related apoptosis-inducing ligand (TRAIL), to render DC direct, potent mediators of tumor cell apoptosis. We demonstrate that the genetic modification of DCs by adenoviral infection to overexpress IL-12, IL-18, and GM-CSF significantly enhanced their ability to stimulate a systemic immune response in the murine breast tumor TS/A following intra-tumoral injection. Furthermore, expression of FasL and TRAIL on DC resulted in a stronger anti-tumor response following intra-tumoral delivery. The anti-tumor effect of different genetically modified-DC was mediated, in part, by the induction of specific cytotoxic T lymphocytes (CTL) activity against the tumor. The genetic modification DC to express one or more immuno-stimulatory molecules was able to augment natural killer (NK) cells and CTL cytolytic activity and interferon-gamma (IFN- $\gamma$ ) production for subsequent immune anti-tumor responses locally as well as systemically. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. Our research is expected to lead Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.

## BODY

### Task 1. Generation of adenoviral vectors, Month 1-6:

Adenoviral shuttle plasmid (Ad-lox) encoding murine B7.1 and CD40L were constructed with standard molecular cloning methods. Then, recombinant adenoviruses propagated by homologous recombination in CRE8 cells of  $\Psi$ 5 DNA (an Ad5-derived, E1- and E3-deleted adenoviral backbone) and shuttle plasmid. Viruses were purified over three consecutive CsCl gradients, dialyzed at 4°C against sterile storage buffer, and stored -80°C. Recombinant adenoviruses were tittered on 293 cells using standard plaque forming unit (PFU) assay (9).

### Task 2. Adenovirus-mediated transduction of BmDC, Month 4-12:

Bone marrow-derived DC was generated from 6-8 week old age female Balb/C mouse as described previously (10). Bone marrow from the tibia and femur of the mouse was collected and passed through a nylon mash. Erythrocytes were lysed with  $\text{NH}_4\text{Cl}$  buffer, and lymphocytes depleted using a cocktail of antibodies (RA3-3A1/6.1, antiB220; 2.43, anti-Lyt2; GK1.5, and anti-L3T4; all from ATCC, Rockville, MD) and rabbit complement on day 0. Remaining cells were cultured for 24 hours in complete media. The non-adherent cells were placed in fresh media containing 1000 U/ml of rmGM-CSF and rmIL-4 on day 1. Cells were cultured for 4 days and harvested for transduction on day 4.

For infection,  $1 \times 10^6$  DC was collected in the round bottomed-tube. The appropriate viruses (50 MOI) including Ad-GM-CSF, Ad-IL-12, Ad-B7.1, Ad-CD40L, and Ad-eGFP, in 1 ml of serum free media were added into the tube and mixed with cells. After incubation for 2 hr in room temperature, fresh media was added and incubated for 20 hr. On day 5, the cultured media of infected DC was replaced with media containing rmGM-CSF and rmIL-4. DC was remained for 2 days and then stained with antibodies for phenotypic analysis, or in vitro and in vivo experiments.

The efficiency of adenoviral transduction was evaluated by percentage of the cell showing eGFP gene expression in Ad-eGFP-infected DC (Fig. 1). Approximately, 70-80% of DC was infected with 50 MOI of adenovirus and expressed transgene. Transgene expression was also quantified by measurement of cytokine secretion from infected DC (Fig. 2). A million of DC infected with 10 or 50 MOI of adenoviruses, produced for up to 8 ng/ml of cytokines for 24 hr. For further phenotypic analysis after adenoviral infection, DC were stained with PE- or FITC-conjugated monoclonal antibodies specific for murine surface molecules such as CD11b, CD11c, CD80, CD86, Gr-1, H-2K, and I-A. Then, expression patterns of surface molecules were determined using FACS analysis. The delivery of Ad-GM-CSF up-regulated CD11c, CD80, and CD40 compared to Ad-LacZ-delivered control (Fig. 3).

Ad-GM/CSF-infected DC was also tested whether the chemokine receptors could be changed to migrate to the lymph node after GM/CSF delivery. The transduced DC was harvested 2 days post infection and the DC suspension was loaded on the upper chamber of membrane-derived well, while in the lower chamber was filled with the media containing MIP-1a or MCP-1, or none of them. After incubation for 4 hr, the number of cell in the lower chamber was counted (Fig. 4). According to enhancement of migration attracted by MIP-1a and MCP-1, GM/CSF expression seemed to yield up-regulation of CCR2 and CCR7 chemokine receptors on the surface of the transduced DC.

### **Task 3. Establishment of animal model and gene delivery in vivo, Month 10-18;**

TS/A cells, an aggressive and poorly immunogenic tumor line derived from Balb/C breast adenocarcinoma, were maintained in RPMI1640 media. Female 6-8week-old Balb/C mice were injected intradermally with  $2 \times 10^5$  TS/A cells on day 0. On day 7, palpable tumor was detected on the site of flank injected with the cells. On day 7, a million of transduced DC with Ad-GM/CSF, Ad-IL-12, Ad-CD40L, Ad-B7.1, or CD40L, significantly suppressed growth of the established tumor (Fig 5 and Fig. 6). Furthermore, Ad-CD40L induced IL-12 secretion from the transduced DC and showed efficient anti-tumor immune responses (data not shown). After an injection of DC/CD40L on day 7, established TS/A tumor gradually decreased and completely disappeared in six out of ten treated mice (Fig. 6). Re-challenged CT26 cells formed tumor on the other side of flank (data not shown).

### **Task 4. Optimization of Dendritic cell function for anti-tumor immunity, Month 13-18:**

- a. Screening genes for achievement of enhancing the activity on DC.
- b. Analysis changing of surface markers with transgene expression on DC by flow cytometry.
- c. Test of cytokines production from genetically-modified dendritic cells.
- d. Migration assay of DCs for specific chemokines.

DC derived from mouse bone marrow exhibited the veiled dendrite morphology typical for DC and displayed a characteristic set of DC surface marker (Table 1). The genetically modified DCs expressed high levels of the MHC class I and II molecules, the costimulatory molecules B7.1 and B7.2, ICAM-1 adhesion molecule, and integrin CD11c. Infection of DC with Ad-IL-12, Ad-GM/CSF, and Ad-CD40L at a 50 MOI resulted in a marked increase in levels of expression of B7.1, B7.2 and MHC class I molecules. For example, 52.6% of CD40L-, 46% of IL-12-, and 47% of Ad-GM/CSF- transduced DC expressed CD86 molecules in comparison with 24.6% and 26.9% in non-transduced and Ad-psi5 control virus-transduced cells, respectively.

Cytokine production from the genetically modified DCs and migration ability of DCs for specific chemokines were measured as described in the last annual report.

#### **Task 5. Evaluation of anti-tumor effect with DCs administration in breast tumor,**

##### **Month 19-24:**

- a. Intra-tumoral injection of genetically-modified dendritic cells on tumor mice.
- b. Measurement of tumor growth with DC administration.

TS/A cells, an aggressive and poorly immunogenic tumor line derived from a Balb/C breast adenocarcinoma, were maintained in RPMI1640 media. Female 6-8 week-old Balb/C mice were injected intra-dermally with  $2 \times 10^5$  TS/A cells on day 0. On day 7, palpable tumor was detected on the site of flank injected with the cells. On day 7, a million of transduced DC with Ad-GM/CSF, Ad-IL-12, Ad-CD40L, Ad-B7.1, or Ad-eGFP, or non-transduced DC were injected intra-tumorally. Tumor growth was determined every 3 days by measuring the long and short diameter of tumor mass with Burnier calipers. The treatment of genetically engineered DC expressing the cytokines such as GM/CSF, IL-12, IL-10 (Fig 7), surface molecules such as B7.1, CD40L (Fig 8), and TNF family such as TRAIL and 4-1BBL (Fig 9) significantly suppressed growth of the established breast tumor. Furthermore, Ad-CD40L induced IL-12 secretion from the transduced DC and showed efficient anti-tumor immune responses (data not shown). Moreover, combined injection with DC/GM-CSF+CD40L and DC/IL-12+B7.1 yielded efficient growth inhibition of the breast tumor (Fig 10). After an injection of DC/CD40L on day 7, established TS/A tumor gradually decreased and completely disappeared in six out of ten treated mice (Fig 8A). Re-challenged TS/A tumor was rejected in the CD40L-treated mice, while the challenged CT26 cells formed tumor on the other side of flank (data not shown).

#### **Task 6. Analysis and determination of mechanisms in anti-tumor effects of modified BmDC, Month 25-**

##### **32.**

- a. Characterization of immune cells infiltrating tumors and surrounding tissues.
- b. Test cultured lymphoid cells for cytotoxic T cell responses against specific breast tumor cells.
- c. Profiling of cytokine production from lymphoid cells against tumor cells.

To test CTL responses against specific TS/A breast tumor in vivo, splenocytes were harvested and pooled from mice, 7 days after intra-tumoral injection with BmDC's and restimulated in vitro with the irradiated (6000 rad) TS/A at a responder to stimulator ratio of 10:1 for 5 days in culture medium supplemented

with 10 IU/mL of IL-2. On day 6, restimulated splenocytes were used as effectors for cytolytic activity against TS/A, YAC-1, and MethA. A million of these target cells were labeled with 100 uCi of  $\text{Na}_2^{51}\text{CrO}_4$  (Amersham, Arlington Heights, IL) for an hour. The restimulated splenocytes were mixed in graded doses with  $10^4$  labeled target cells in 200 uL of tissue culture medium in round bottom 96-well tissue culture plates. Cells were incubated for 4 hr at 37°C in a 5% CO<sub>2</sub> humidified incubator. The release of  $^{51}\text{Cr}$  was measured with a gamma counter, and the percent of specific lysis was calculated as follows:  $100 \times [(\text{experimental release} - \text{spontaneous release}) / ((\text{maximum release} - \text{spontaneous release}))]$ . Spontaneous and maximal release was determined in the presence of either medium or 10% SDS. Genetically-modified DC to express B7.1 (Fig. 11-A), GM-CSF (Fig. 11-B), IL-12 (Fig. 11-C), CD40L (Fig. 11-D), or TRAIL (Fig. 11-E) induced strong CTL responses against specific TS/A tumor as high as 80% cytotoxicity. However, both of therapeutic gene- and lacZ control gene-treated group showed minimal level of CTL responses, which were similar with activities from non-infected DC or saline treated group, against YAC1 non-specific NK-sensitive cells. Moreover, combined injection with DC/GM-CSF+CD40L and DC/IL-12+B7.1 yielded efficient growth inhibition of the breast tumor (Fig 10) and CTL from DC/GM-CSF+CD40L showed strong cytotoxicity against target TS/A tumor cells (Fig. 12).

Secretion of cytokines was measured from the cultured-lymphoid cells with stimulation either specific or non-specific tumor. 7 and 14 days post injection of genetically-modified DC into the tumor site, draining lymph nodes and spleen were pooled from the mice and lymphocytes or splenocytes obtained cultured at  $5 \times 10^5$  cells per well in triplicate either with or without irradiated TS/A specific tumor or 4T1, breast tumor cells for 2 days. Culture supernatant was collected and was subjected for analysis of cytokine production such as IFN-gamma (Fig. 13), IL-4 (Fig. 14), and IL-10 (Fig. 15). Cytokine production was detected on the antibody-coated plates. Splenocytes from the mouse treated with CD40L secreted high amount of IFN-gamma with TS/A tumor stimulation as well as without stimulation. This result was consistent with the result of CTL response and regression of tumor growth.

#### **Task 7. Final analysis and establishment of protocol for breast cancer therapy, Month 29-36:**

- a. Evaluation of anti-tumor immunity acquired from administration of genetically modified DC in breast tumor.
- b. Initiate preparation of protocol for clinical trial using genetically-modified dendritic cell immunotherapy for treatment of breast cancer.

Induction of anti-tumor immunity was evaluated in the DC-injected mouse bearing TS/A tumor. TS/A tumor cells were injected into right flank of BALB/c mouse on day 0 and into the left flank on day 3. A week later, either non-modified DC or genetically modified DC to express CD40L or eGFP was injected into the established tumor in right side of flank. Size of tumor in both flaks was measured in every 3 days. As shown in Fig. 16, tumor growth was suppressed in the DC/CD40L-injected tumor as well as its contra-lateral, non-injected tumor compared to control group. To test establishment of stable anti-tumor immunity, secondary TS/A tumor cells were injected into flank of the tumor free mouse, which were injected DC/CD40L into tumor site. Re-challenged TS/A tumor was rejected in the CD40L-treated mice, while the challenged CT26 cells formed tumor on the other side of flank (Table 2).

**Table 1. Phenotyping of DCs transduced with adenoviruses.**

DCs	CD11c	CD80	CD86	Iad	CD40
Control	76%	46%	20%	80	26%
Ad/lacZ	74%	56%	27%	78	32%
Ad/IL-10	84%	74%	37%	80	28%
Ad/IL-12	82%	72%	46%	82	33%
Ad/GM.CSF	80%	74%	47%	84	31%
Ad/CD40L	78%	64%	53%	87	52%

**Table 2. Tumor formation with secondary challenge**

Tumor		Number of mice bearing tumor
TS/A	CD40L	0/3
	control (primary)	3/3
CT26	CD40L	2/3
	control (primary)	3/3

## **KEY RESEARCH ACCOMPLISHMENTS**

Generation of Bone marrow-derived dendritic cell from Balb/C mouse  
Construction of adenoviral vectors encoding mouse B7.1, TRAIL, 4-1BBL, and CD40L  
Propagation of adenoviruses by recombination in the CRE8 cells  
Purification of adenoviruses by CsCl gradients  
Transduction of DCs with adenoviruses  
Evaluation of transduction efficiency using eGFP transgene expression  
Evaluation of transgene expression by measurement of cytokine secretion  
Phenotypic analysis of the transduced using flow cytometry  
Migration assay against specific chemokines  
Induction of TS/A breast tumor in Balb/C mouse model  
Injection of the transduced DCs intra-tumor site in established tumor  
Measurement of tumor size  
Evaluation of anti-tumor effect with DCs injection  
Re-challenge of TS/A or non-specific syngenic tumor in the cured mouse  
Combined gene delivery using DC into breast tumor  
Isolation of splenocytes from tumor bearing mouse  
Co-culture of splenocytes with specific or non-specific irradiated tumor  
Analysis of cytotoxic T cell responses against breast tumor.  
Analysis of cytokine production from tumor-stimulated splenocytes.  
Challenge of tumor in the contra-lateral flank of tumor-bearing mice.  
Evaluation of anti- tumor immunity with non-treated tumor.

## **REPORTABLE OUTCOMES**

Construction of adenoviral vectors encoding mouse B7.1, TRAIL, 4-1BBL, and CD40L  
Generation of adenoviruses including Ad/GM-CSF, Ad/IL-12, Ad/IL-10, Ad/CD40L, Ad/B7.1, Ad/TRAIL, Ad/4-1BBL, and Ad/eGFP in large scale  
Abstracts for the 4<sup>th</sup> annual meeting of American society of gene therapy (appended)  
Abstracts for the 5<sup>th</sup> annual meeting of American society of gene therapy (appended)  
Abstract for the 'ERA of HOPE' meeting (Sep. 2002)

## CONCLUSIONS

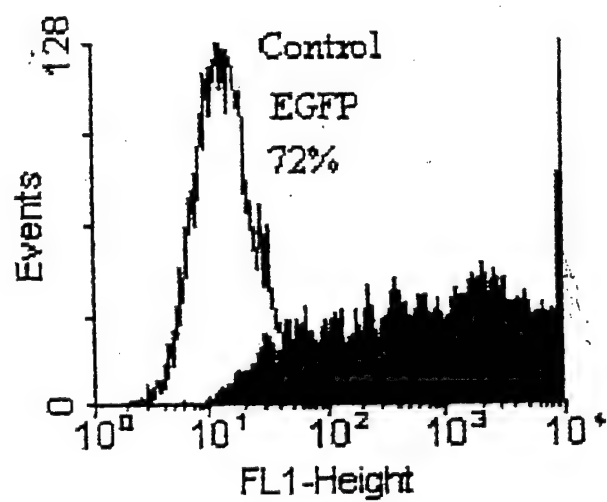
1. Bone marrow-derived dendritic cells (BmDC) were infected 50 MOI of adenoviruses encoding potent therapeutic genes. Surface molecule genes were delivered to 75 ~ 98 % of total DC. A million of DC which was infected with Ad-cytokines secreted 15 ~ 40 ng/ml of cytokines in the culture media for 48 hours.
2. Each dendritic cell could be infected with two or more different type of adenoviruses. Even though adenoviral infection caused slight higher levels of MHC and co-stimulatory molecules on DC surface, double infection might not increase the level of surface molecules
3. Intra-tumoral injection of DC/CD40L and DC/IL-12 showed the significant suppression of growth in TS/A breast tumor model. Moreover, combined injection with DC/GM-CSF+CD40L and DC/IL-12+B7.1 yielded efficient growth inhibition of the breast tumor.
5. Splenocytes from the DC/CD40L- or DC/IL-12- injected mouse produced higher level of IFN-g with stimulation of the irradiated TS/A tumor cell. In contrast, Th2 type cytokines such as IL-4 and IL-10 were maintained in low level in regardless of type or number of genes, or DC injection (data not shown).
6. We demonstrated that administration of the genetically engineered DC showed the anti-tumor effect on the established breast tumor model. We suggested the possible treatment of tumor with combined gene delivery mediated by DC.
7. Genetically-modified DC to express B7.1, GM/CSF, IL-12, CD40L, or TRAIL induced strong CTL responses *in vivo* against specific TS/A tumor as high as 80% cytotoxicity.
8. Combined gene delivery resulted in strong CTL responses as high as single therapeutic gene delivery. However, synergic activity was not detected in the combination of GM/CSF & CD40L and GM/CSF & TRAIL.
9. Splenocytes from the mouse treated with CD40L secreted high amount of IFN-gamma with TS/A tumor stimulation as well as without stimulation but not with YAC1 non-specific cells.

10. Combined gene delivery of GM/CSF & CD40L and GM/CSF & TRAIL showed the similar level of IL-4, IL-10, and IFN-gamma secretion with single gene delivery.
11. Administration of DC/CD40L suppressed TS/A breast tumor growth in both of injected tumor as well as its contra-lateral, non-injected tumor.
12. Re-challenged TS/A tumor was rejected in the CD40L-treated mice, while the challenged CT26 cells formed tumor on the other side of flank.
13. We demonstrated that administration of the genetically engineered DC showed the anti-tumor effect on the established breast tumor model. We also showed that delivery of genetically modified DC induced strong anti-tumor immune responses in breast tumor model

## REFERENCES

1. Nishioka Y, Hirao M, Robbins PD, Lotze MT, and Tahara H. (1999) Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin 12. *Cancer Res.* 59:4035-4041.
2. Pogador, A., Snyder, D., Gilboa, E. 1996. Induction of antitumor immunity using bone marrow-generated dendritic cell. *J. Immunol.* 156:2918.
3. Labeur, M. S., Roters, B., Pers, B., Mehling, A., Luger, T.A., Schwarz, T., Grabbe, S. 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol.* 162:168-175.
4. Macatonia, S. E., Taylor, P.M., Knight, S.C., Askonas, B.A. 1989. Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. *J. Exp. Med.* 1169:1255-1264.
5. Schuler, G., Steinman R.M. 1986. Dendritic cells as adjuvants for immune mediated resistance to tumors. *J. Exp. Med.* 186:1183.
6. Veerman, M. D., Heirman, C., Meirvenne, S.V., Devos, S., Corthals, J., Moser, M., Thielemans, K. 1999. Retrovirally transduced bone marrow-derived dendritic cells require CD4<sup>+</sup> T cell help to elicit protective and therapeutic anti-tumor immunity. *J. Immunol.* 162:144-151.
7. Tazi, A., Bouchonnet, F., Grandsaigue, M., Bounsell, L., Hance, A.J., Soler, P. 1993. Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers. *J. Clin. Invest.* 91:566-576.
8. Lu, L., Hsieh, M., Oriss, T.B., Morel, P.A., Starzl, T.E., Rao, A.S., Thomson, A.W. 1995. Generation of DC from mouse spleen cell cultures in response to GM-CSF: immunophenotypic and functional analyses. *Immunology.* 84:127-134.
9. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., Phipps, M.L. 1997. Construction of adenovirus vector through Cre-lox recombination. *J. Virol.* 71:1842-1849.
10. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., Steinman, R.M. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocytes/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693-1702.

Figure 1. Transduction Efficiency of Ad-eGFP Infection in BmDC



## Figure 2. Transgene Expression in BmDC Infected with Ad-GM/CSF and Ad-IL-12

A. mouse GM/CSF secretion, B. mouse IL-12 secretion in culture media for 24 hours after adenoviral infection

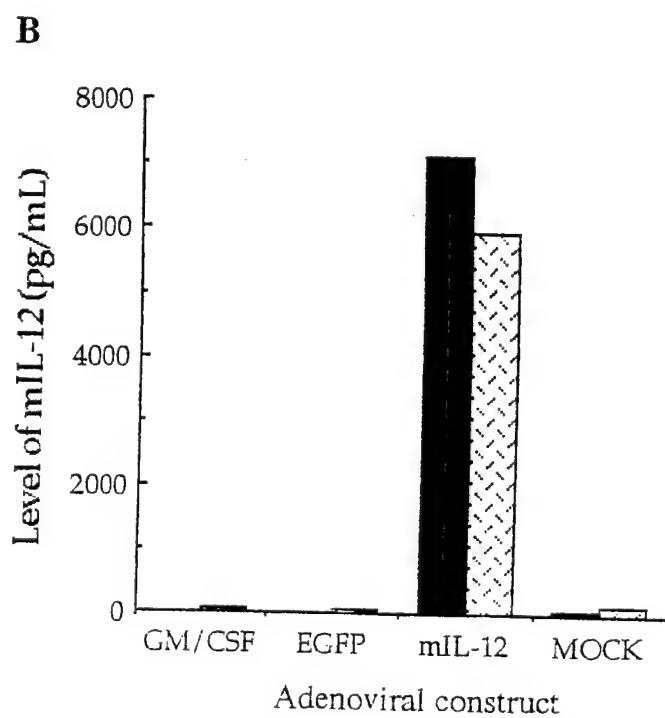
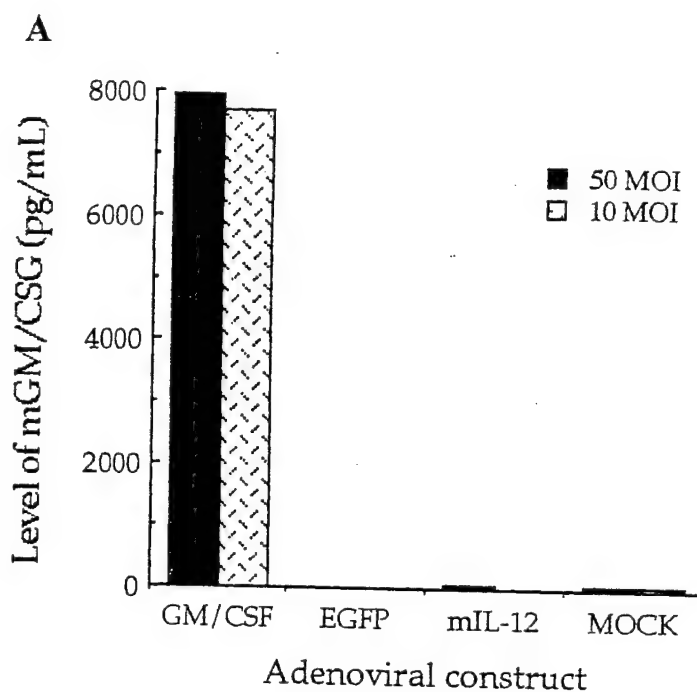
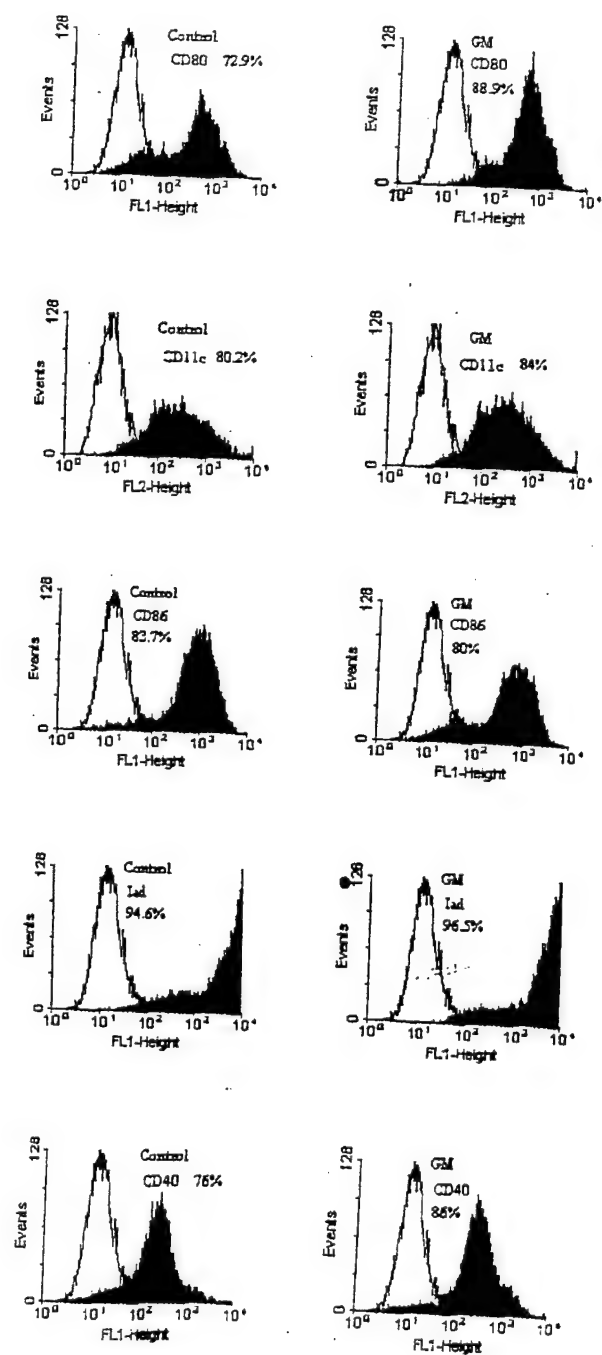
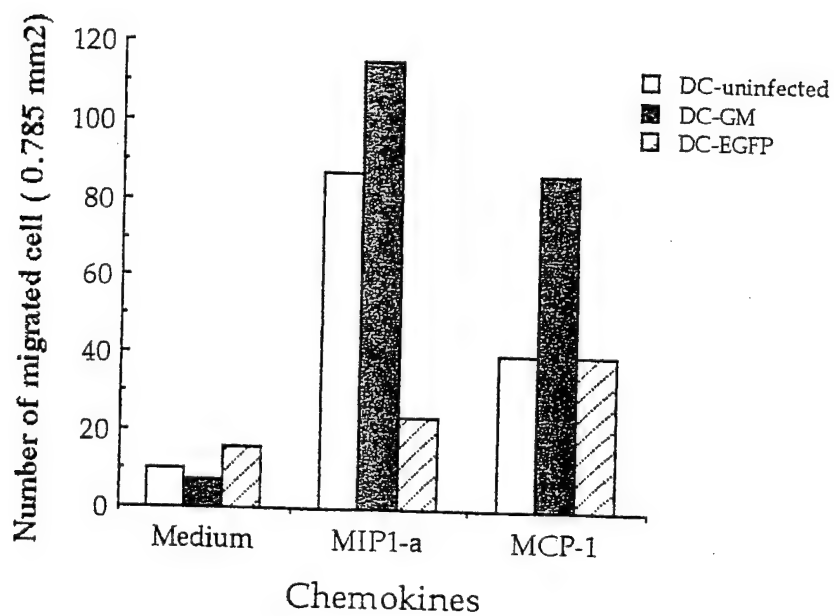


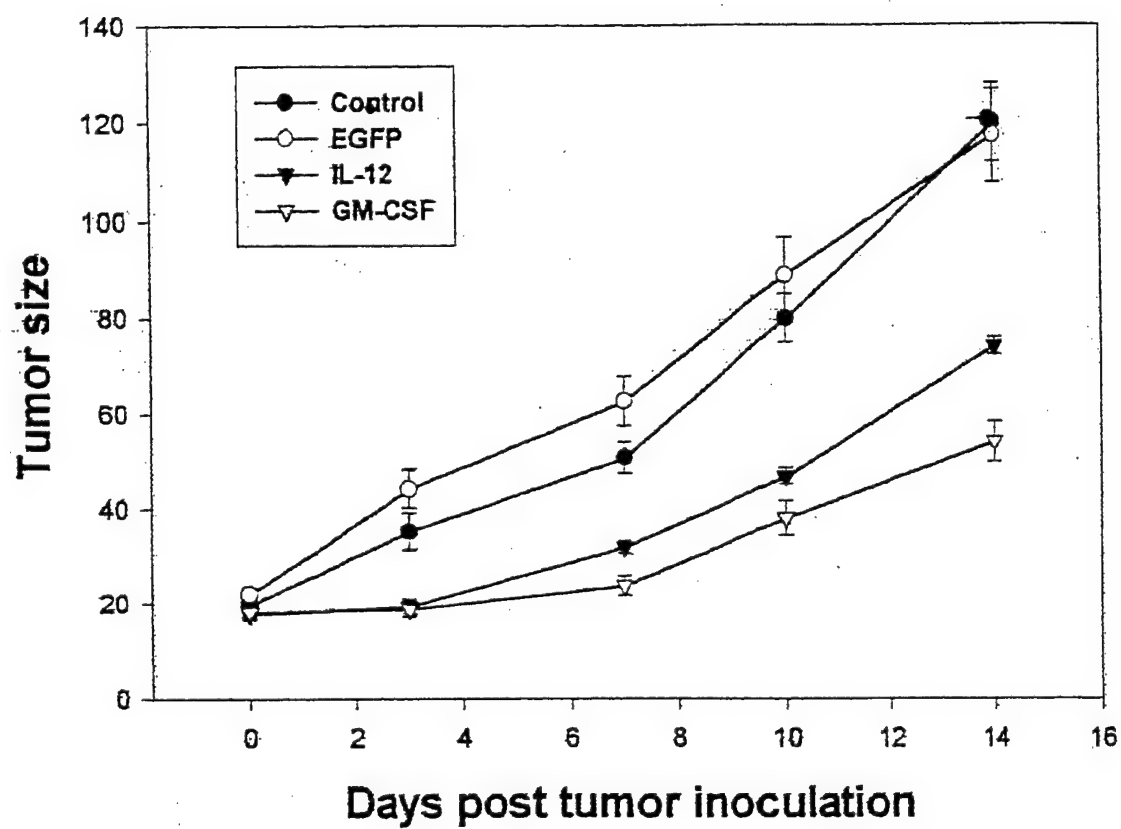
Figure 3. Phenotypic Analysis of BmDC infected with Ad-LacZ (A) and Ad-GM/CSF (B)



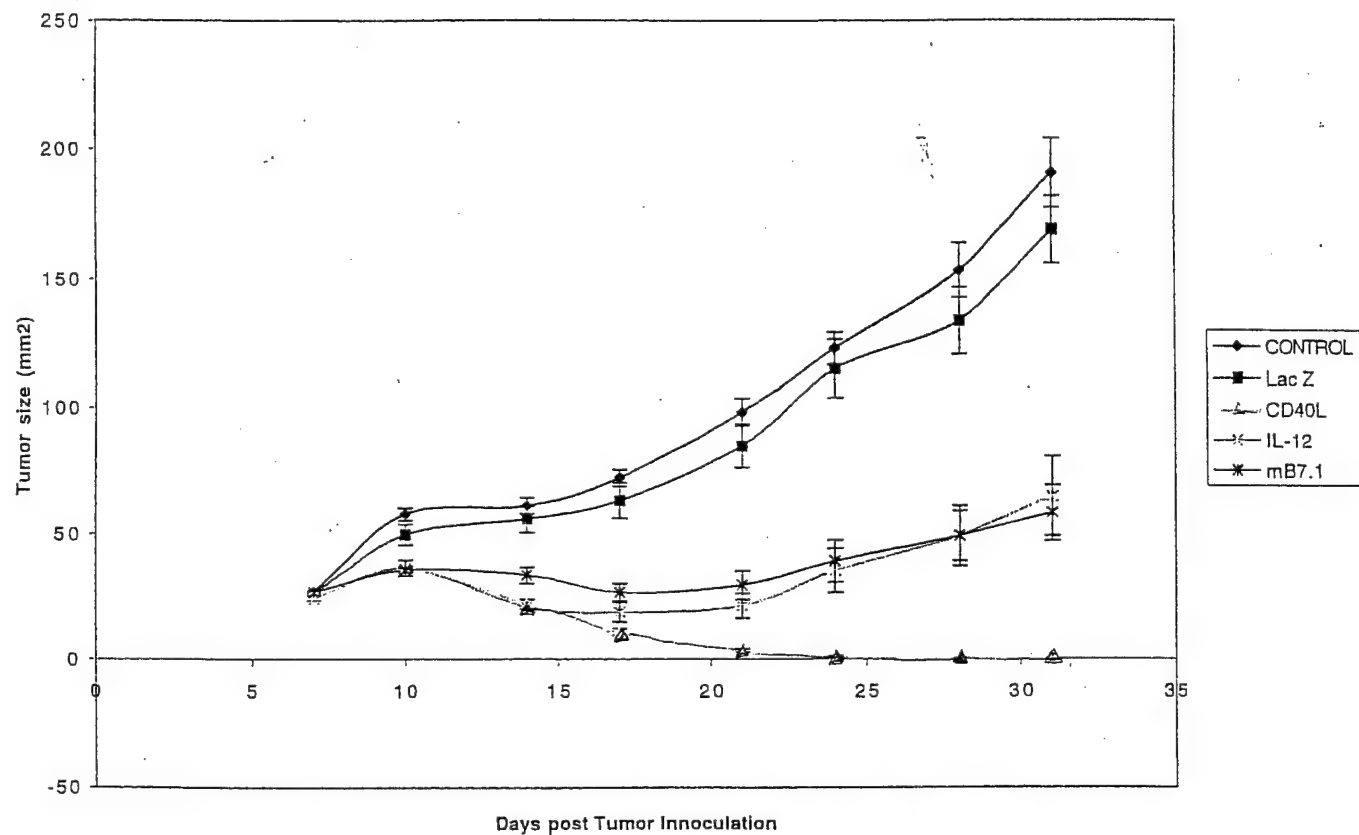
**Figure 4. Migration Analysis of BmDC Infected with Ad-eGFP and Ad-GM/CSF against Chemokines, MIP-1a and MCP1**



**Figure 5. Delayed Growth of Established TS/A tumor after Intra-tumoral Injection of BmDC Transduced with Ad-GM/CSF and Ad-IL-12**



**Figure 6. Suppression of Tumor Growth after Intra-tumoral Injection of BmDC Transduced with Ad-B7.1, Ad-IL-12, and CD40 ligand in Mouse Model Bearing the Established Breast Tumor**



**Figure 7. Administration of DC expressing Cytokines in the established TS/A Breast Tumor of Balb/C Mouse**

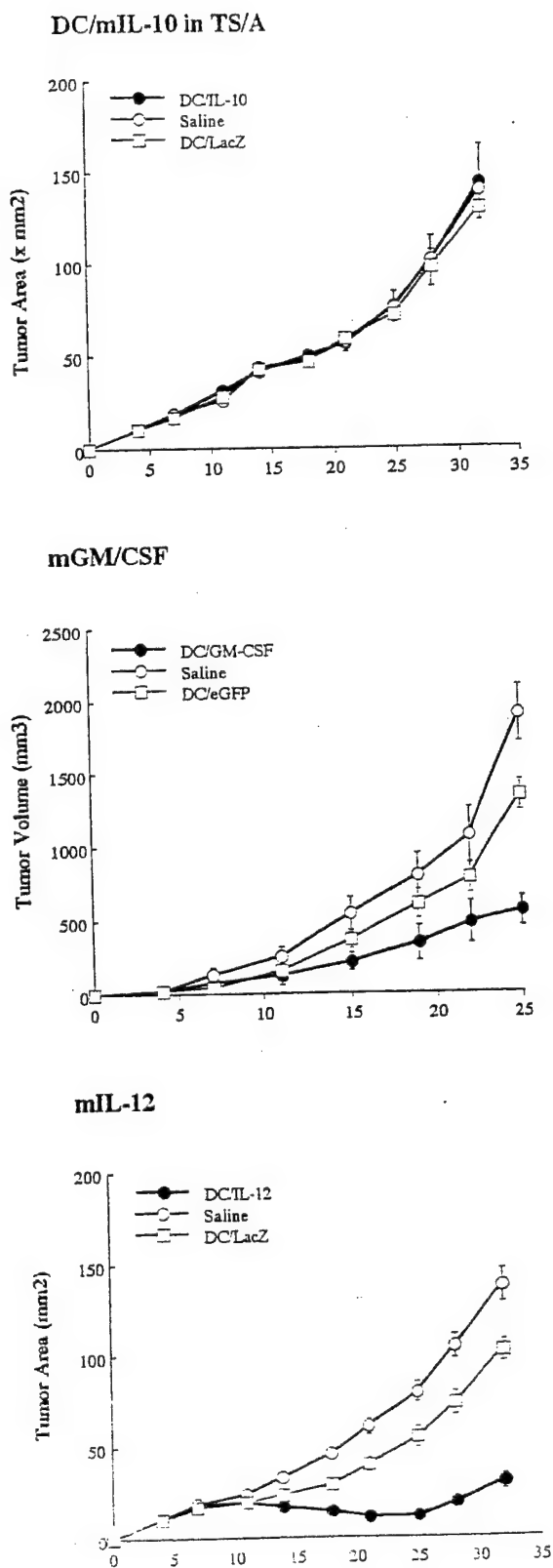
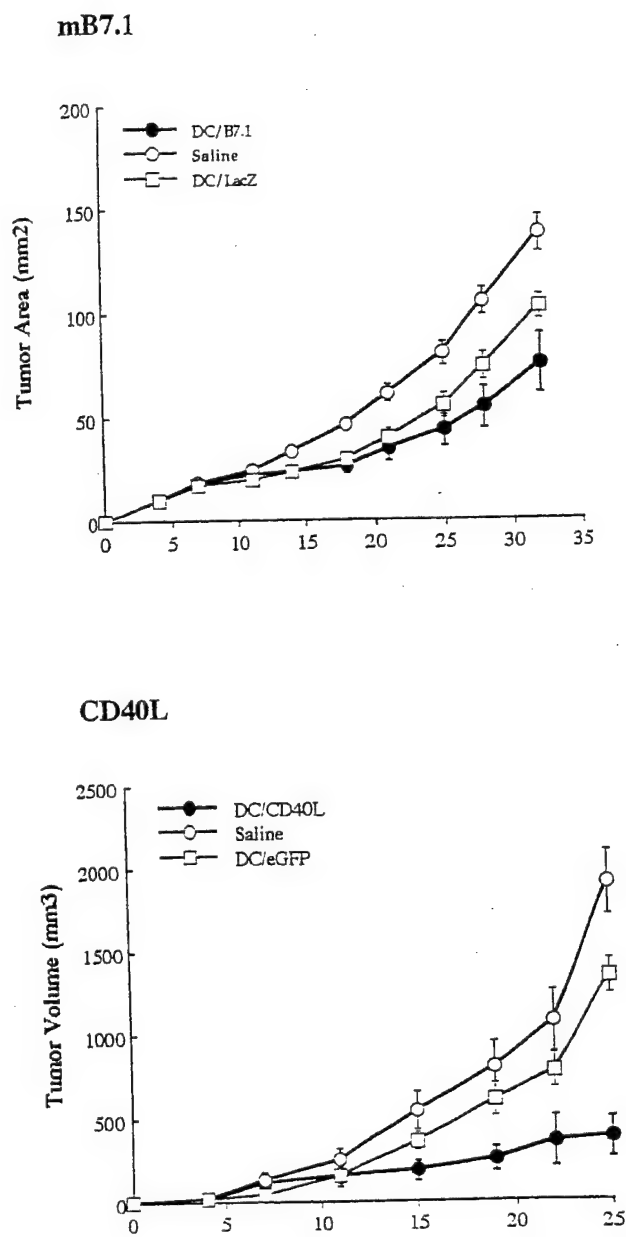
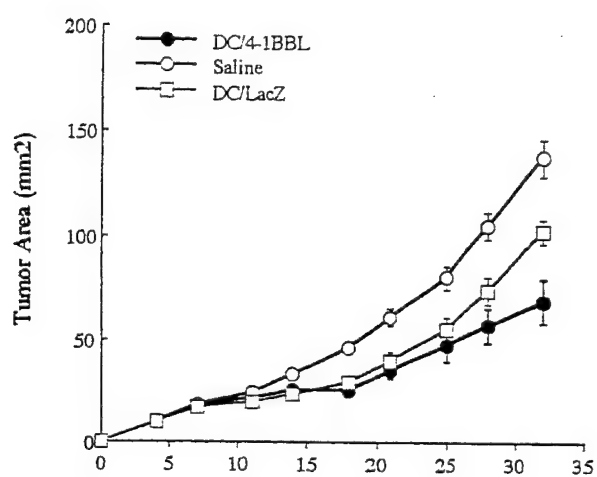


Figure 8. Administration of DC expressing surface molecules in the established TS/A Breast Tumor of Balb/C Mouse

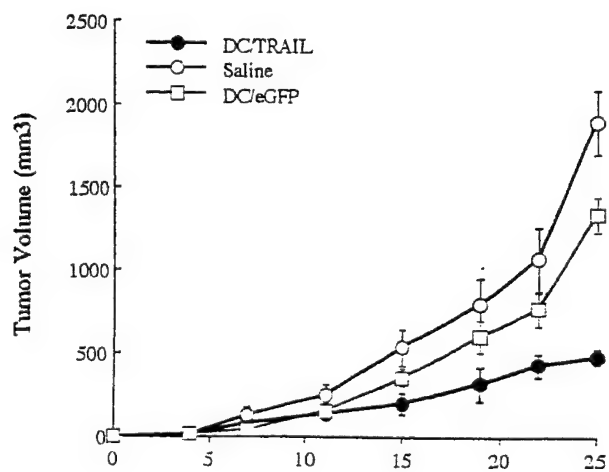


**Figure 9. Administration of DC expressing TNF family into the established TS/A Breast Tumor in Mouse Model**

**4-1BBL**



**TRAIL**



**Figure 10. Combined Gene Delivery using DC to Treat the established TS/A Breast Tumor in Mouse Model**

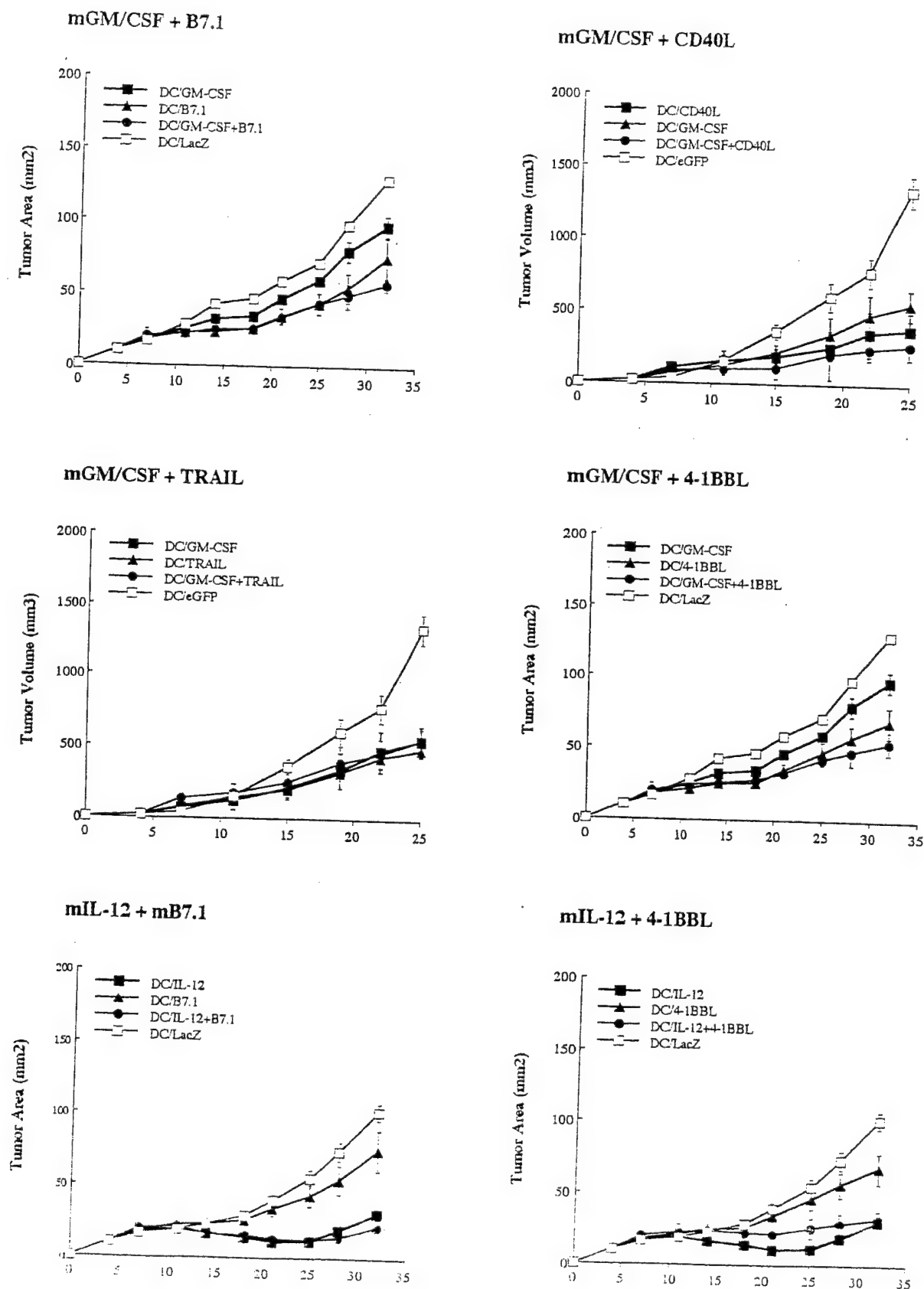


Figure 11. Cytotoxic T Lymphocytes Responses against TS/A breast tumor

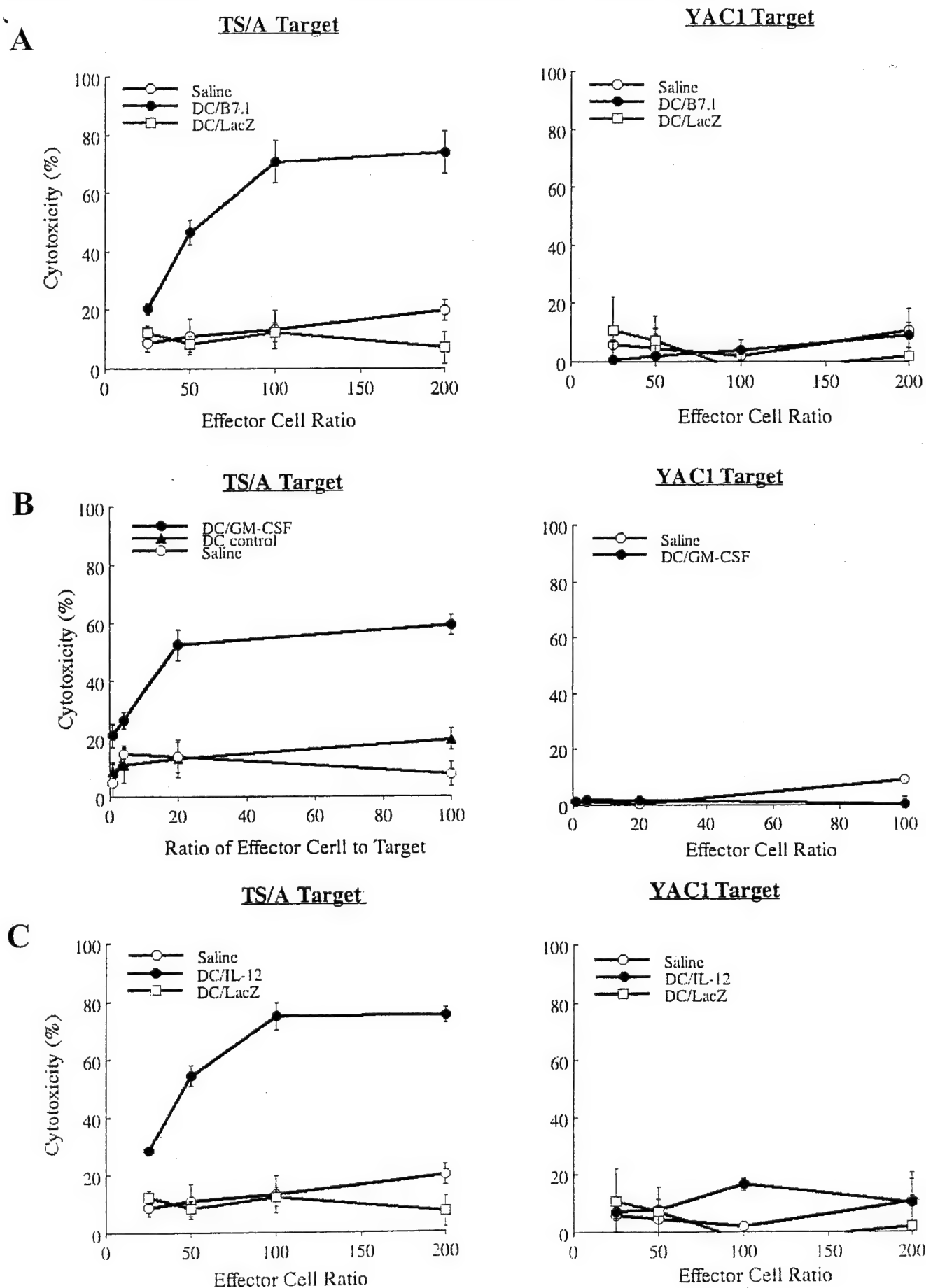


Figure 11. Cytotoxic T Lymphocytes Responses against TS/A breast tumor

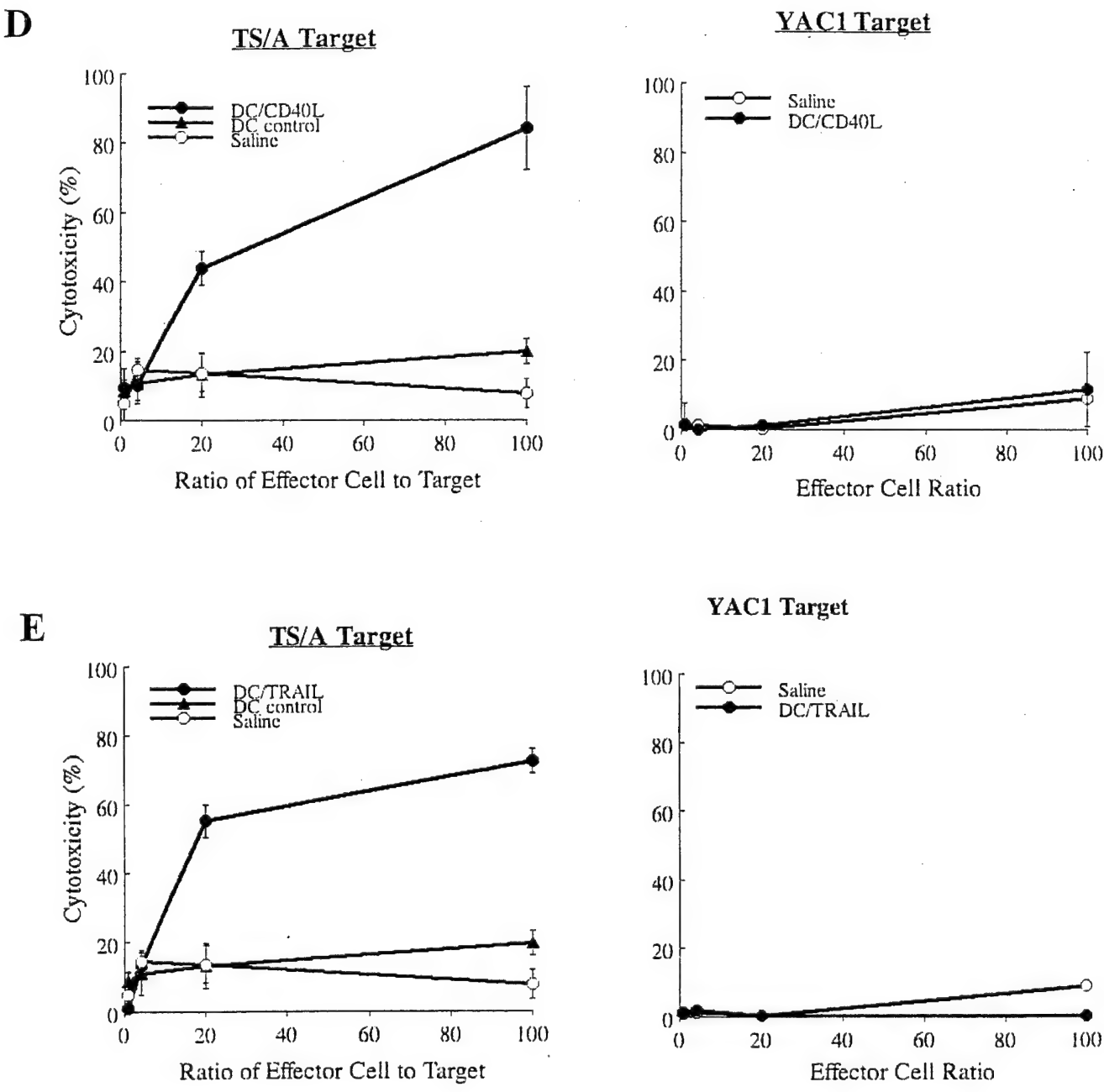
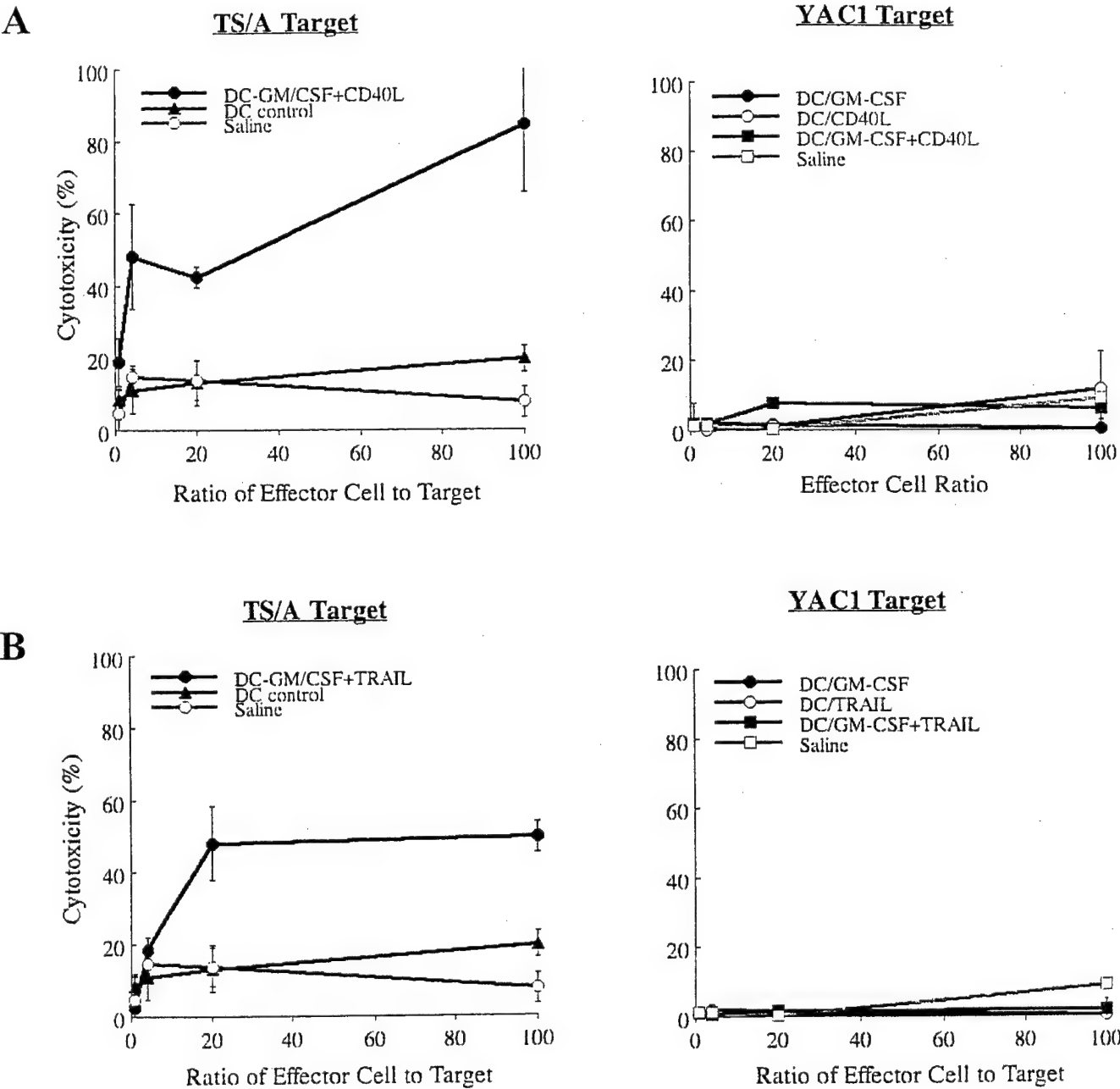
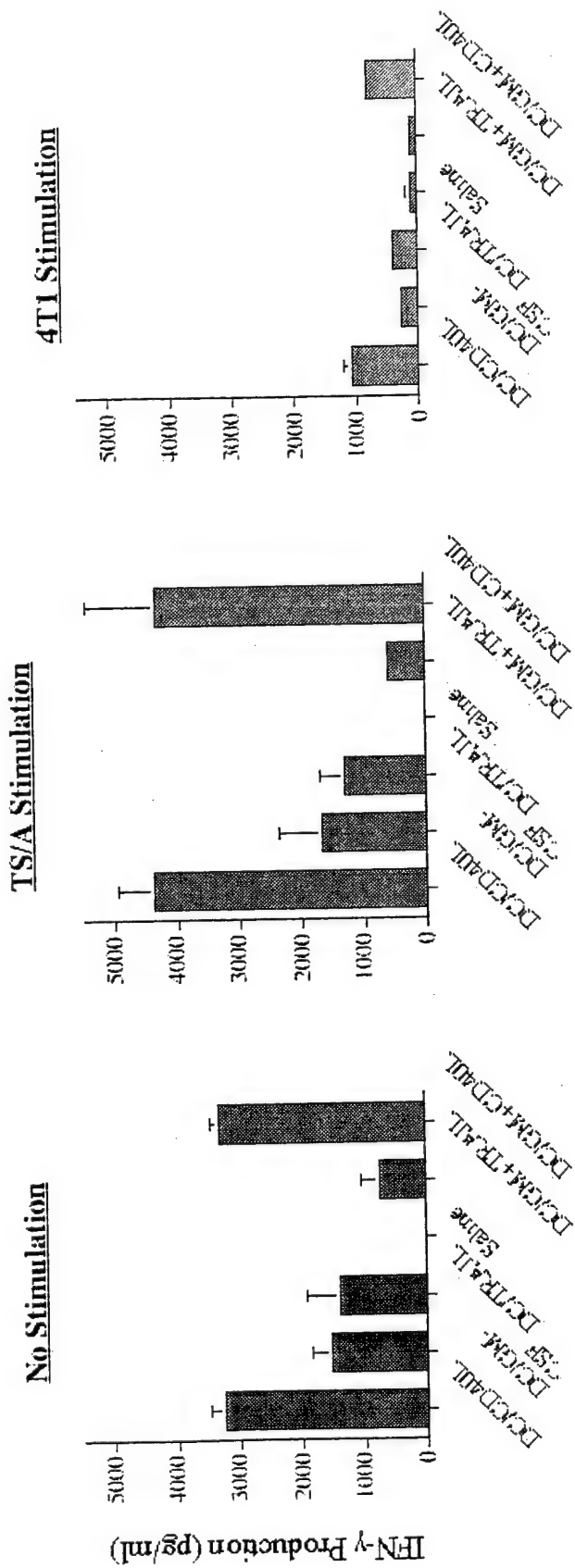


Figure 12. Cytotoxic T Lymphocytes Responses of Combined Gene Delivery



**Figure 13. IFN- $\gamma$  production**



### Figure 14. Interleukin-4 Production

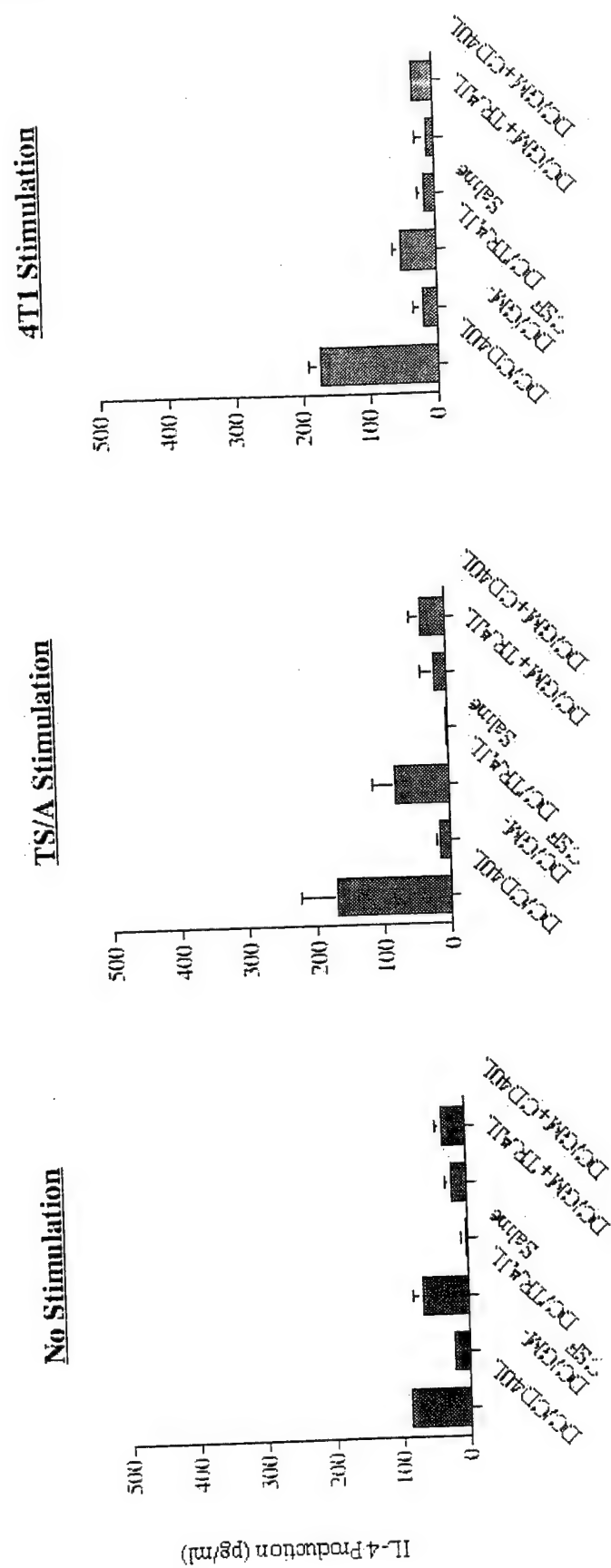


Figure 15. Interleukin-10 Production

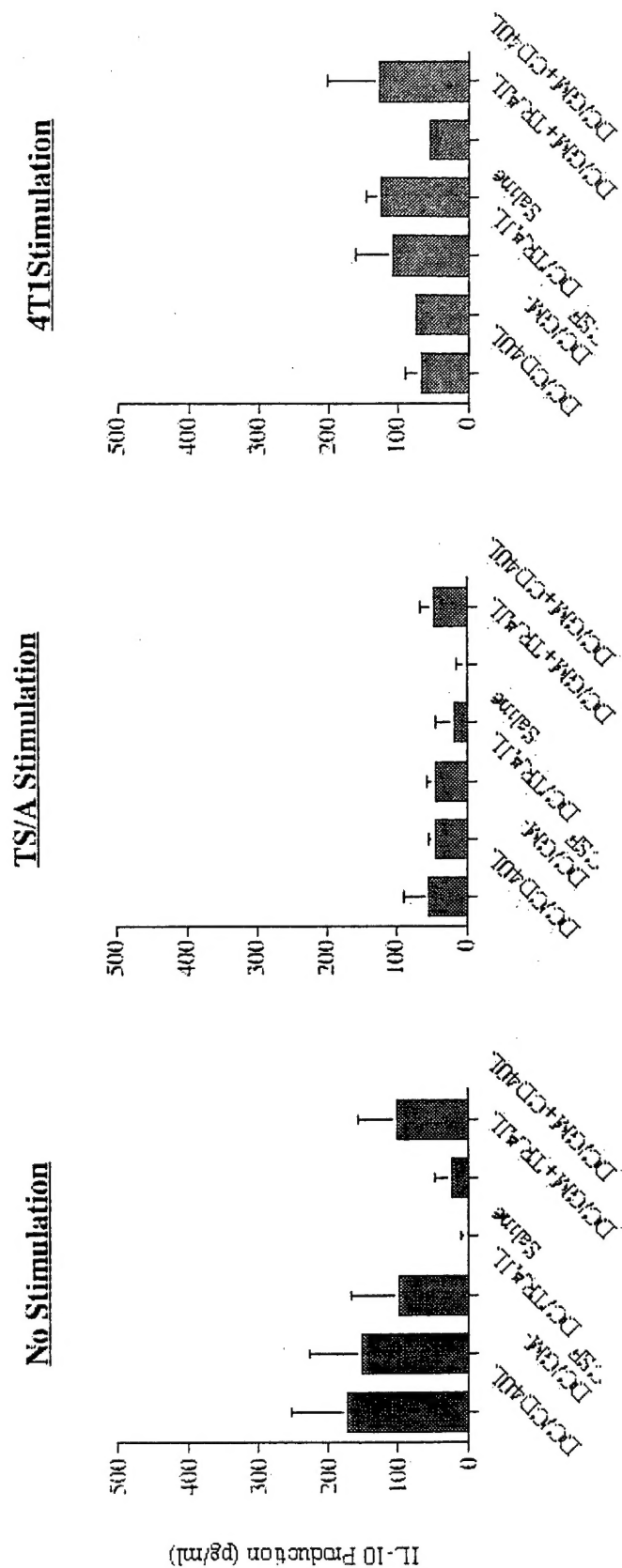
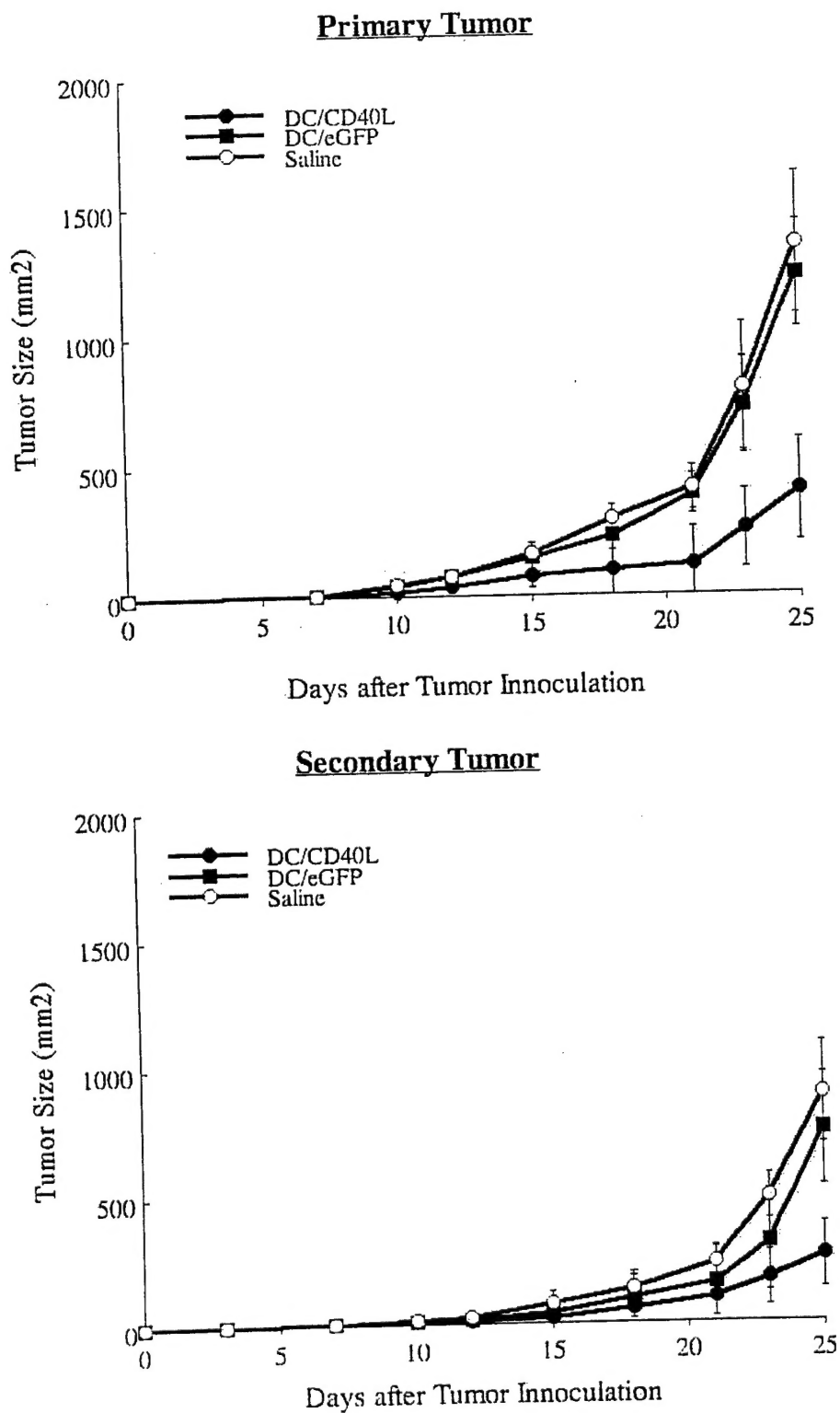


Figure 16. Anti-tumor Immunity of Genetically Modified DC to express CD40L in the treated TS/A as well as non-treated contra-lateral Tumor



## APPENDICS

Abstract for 4<sup>th</sup> Annual Meeting of American Society of Gene Therapy

### 213. Intratumoral Administration of AD-CD40L or DC-CD40L Elicited Effective Antitumor Immunity in Mice

Zoya Yurkovetsky\*, Andrea Gambotto\*, Michael Shurin†, Seon-Hee Kim\*, Paul Robbins\*

\*Departments of Molecular Genetics and Biochemistry

†Pathology, University of Pittsburgh, Pittsburgh, PA 15261

Interaction between CD40 on antigen-presenting cells and its ligand CD40L (CD154) on T cells plays an important role in the induction of immune responses, including anti-tumor immunity. Soluble CD40L and gene transfer of CD40L to tumor cells have been shown to induce specific immune responses in several murine tumor models. In this study, we have evaluated whether expression of CD40L at the site of the tumor elicits an immune response to established tumors in mice. A recombinant adenovirus encoding murine CD40L (Ad-CD40L) was constructed and tested in the MC38 murine colon adenocarcinoma and TS/A murine breast adenocarcinoma models. Administration of Ad-CD40L on Day 7 after tumor inoculation resulted in a significant inhibition of MC38 tumor growth when compared with the control groups treated with either HBSS or control adenovirus. In contrast, intra-tumoral injection of Ad-CD40L did not result in a significant inhibition of TS/A tumor progression. Interestingly, no significant differences between a single or multiple administrations of Ad-CD40L were detected. We also have examined the therapeutic efficacy of intra-tumor injection of murine bone marrow-derived dendritic cells (DC) gene infected with Ad-CD40L. Intra-tumoral injection of DC-CD40L DC-based resulted in a significant inhibition of MC38 tumor growth as compared to control groups treated with non-transfected DC. In addition, treatment of established day 7 TS/A tumors with Ad-CD40L/DC resulted in all animals being tumor-free 20 days post-therapy with induction of systemic immunity. Taken together, our data demonstrate that Ad-CD40L transduction of DC or tumor cells at the tumor site is effective in inducing anti-tumor immunity.

**Combined Immunotherapy of a Murine Mammary Tumor using Genetically Modified Dendritic Cells.** Seon Hee Kim, Zoya R. Yurkovetsky and Paul D. Robbins,  
Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA

We have hypothesized that intra-tumoral injection of immature DCs should result in acquisition of tumor antigens at the site of injection followed by migration to lymph node and spleen. Indeed, we have demonstrated previously that fluorescently labeled DCs are able to migrate to lymph nodes and spleen following intra-tumoral injection and stimulate a tumor specific T cell response. Here we have used adenoviral vectors, able to efficiently infect both murine and human DC, to deliver IL-12, IL-18, and GM-CSF and the costimulatory molecules B7.1 and CD40L, either alone or in combination, to bone marrow-derived DC (BmDC). We showed that the genetic modification of DCs to overexpress IL-12, GM-CSF, and CD40L significantly enhanced their ability to stimulate a systemic immune response following intra-tumoral injection. In addition, we have used adenoviral gene transfer of members of the TNF family able to stimulate apoptosis, FasL and TRAIL, to render DC direct, potent mediators of tumor cell apoptosis. We demonstrate that the genetic modification of DCs by adenoviral infection to overexpress IL-12, IL-18, and GM-CSF significantly enhanced their ability to stimulate a systemic immune response in the murine breast tumor TS/A following intra-tumoral injection. Furthermore, expression of FasL and TRAIL on DC resulted in a stronger anti-tumor response following intra-tumoral delivery. The anti-tumor effect of different genetically modified-DC was mediated, in part, by the induction of specific CTL activity against the tumor. The genetic modification DC to express one or more immuno-stimulatory molecules was able to augment NK cells and CTL cytolytic activity and IFN-gamma production for subsequent immune anti-tumor responses locally as well as systemically. We currently are examining the optimal combination of genes in order to induce the most effective anti-tumor response following intra-tumoral infection of the genetically modified DC. Our research is expected to lead Phase I clinical trial to assess the feasibility and efficacy of treating and preventing breast cancer by intra-tumor injection of genetically modified DCs.